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Thiorhodococcus mannitoliphagus sp. nov., a purple sulfur bacterium from the White Sea

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A novel purple sulfur bacterium, strain WS^T, was isolated from a microbial mat from an estuary of the White Sea. Individual cells are coccoid shaped, motile by flagella and do not contain gas vesicles. The mean cell diameter is 1·85 μm (range 1·5–2·0 μm). Cell suspensions exhibit a purple–violet colour. They contain bacteriochlorophyll *a* and carotenoids of the rhodopinal series as photosynthetic pigments. The novel bacterium is an anoxygenic photoautotroph, using sulfide, thiosulfate, sulfite and elemental sulfur as electron donors for photosynthesis and is capable of photoassimilating several organic carbon sources in the presence of carbonate and a reduced sulfur source (sulfide and/or thiosulfate). Sulfur globules, formed during oxidation of sulfide, are stored transiently inside the cells. Optimal salinity and pH for growth are at 0·5–2·0 % NaCl and pH 7·0–7·5. The DNA base composition of strain WS^T is 61·8 mol% G+C. 16S rRNA gene sequence analysis showed that the new isolate belongs to the genus *Thiorhodococcus*, with *Thiorhodococcus minor* CE2203^T as the nearest relative (sequence similarity of 97·3 %). Several distinct differences from described species necessitate the description of a novel species. *Thiorhodococcus mannitoliphagus* sp. nov. is the proposed name, with strain WS^T (=ATCC BAA-1228^T=VKM B-2393^T) as the type strain.

Along marine shorelines, purple sulfur bacteria are often found in anoxic sediments and in shallow waters of separated water bodies, such as coastal lagoons. Prerequisites for their development are the presence of both reduced sulfur compounds and solar light. Because these factors naturally form counter-current gradients, growth of phototrophic bacteria is often found within thin layers along the appropriate borderline. These special conditions arise for example regularly in estuaries, where so-called microbial mats develop that exhibit very high population densities in thin coloured layers (van Gemerden & Mas, 1995). Due to the transient situation in estuaries between freshwater and marine salt concentrations, both marine and freshwater species of phototrophic bacteria can be found (Gorlenko *et al.*, 1985; Puchkova *et al.*, 2000).

Purple sulfur bacteria belong to the *Gammaproteobacteria* and comprise the two families *Chromatiaceae* and *Ectothiorhodospiraceae* (Imhoff, 1984). These families differ in respect to the deposition of sulfur globules formed during the oxidation of reduced sulfur compounds: members of the *Chromatiaceae* deposit the globules intracellularly, whereas representatives of the *Ectothiorhodospiraceae* deposit them extracellularly. Phenotypic characteristics like cell shape or pigment composition were traditional criteria to distinguish bacterial species (Winogradsky, 1888; Pfennig & Trüper, 1974). Today, the analysis of 16S rRNA gene nucleotide sequences is an additional important tool for species differentiation (Imhoff & Süling, 1996; Imhoff *et al.*, 1998a, b). It is particularly valuable if combined with phenotypic properties to allow a detailed characterization of new bacterial isolates.

Strain WS^T was isolated from material taken from a microbial mat in an estuary of the Nilma river, on the White Sea coast of north-western Russia. In this area, regions of shallow supralittoral beach, which are covered only periodically by brackish water, exhibit the development of natural microbial mats. In addition to cells similar in morphology to strain WS^T, the purple sulfur bacteria *Thiocapsa roseopersicina*, *Thiocapsa purpurea*, *Thiocapsa litoralis*, *Thiorhodovibrio winogradskyi* and *Allochromatium vinosum* and the green sulfur bacterium *Prosthecochloris aestuarii* were found in these microbial mats (Gorlenko *et al.*, 1985; Puchkova *et al.*, 2000).

The 16S rRNA gene sequence of strain WS^T exhibited clear affiliation to the genus *Thiorhodococcus*. The genus *Thiorhodococcus*, with the type species *Thiorhodococcus minor* (Guyoneaud *et al.*, 1997; Imhoff *et al.*, 1998b), is one of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WS^T is AJ971090.

the genera belonging to the family *Chromatiaceae*. Important morphological features such as coccoid cells are common to the genera *Thiorhodococcus*, *Thiocystis*, *Thiocapsa*, *Thiococcus*, *Thiohalocapsa*, *Thiolamprovum*, *Lamprocystis*, *Thioalkalicoccus* and *Thioflavicoccus* (Guyoneaud *et al.*, 1997; Imhoff, 2001; Imhoff & Pfennig, 2001; Bryantseva *et al.*, 2000). Both the sequence distance and a number of differences in phenotypic properties differentiate the novel bacterium from known *Thiorhodococcus* species, which consequently necessitates the description of a novel species.

For isolation of strain WS^T, cultivation of the pure culture and for growth experiments, Pfennig's medium was used (Pfennig & Trüper, 1992) (l^{-1}): 0·34 g KH_2PO_4, 0·34 g NH_4Cl, 0·5 g $MgSO_4.7H_2O$, 0·05 g $CaCl_2.2H_2O$, 0·34 g KCl, 1 ml SL A (Imhoff, 1992), 20 μg vitamin B_{12}, 1·5 g $NaHCO_3$, 0·4 g $Na_2S.7–9H_2O$, 0·5 g $Na_2S_3O_3.5H_2O$, 15 g NaCl, 2·5 g $MgCl_2.6H_2O$. The pH was adjusted to 7·5.

Pure cultures were obtained by repeated application of the deep-agar dilution method (Pfennig & Trüper, 1992). Agar tubes were incubated at 30 °C under a light–dark cycle (16 h 500 lx light, 8 h dark) using tungsten lamps. Purity of the isolate was checked by both microscopy and growth tests in deep-agar or liquid media supplemented with 5 mM acetate and incubated in the dark. Pure cultures were grown in 100 ml screw-capped bottles filled with synthetic medium, incubated at 2000 lx (42 μmol quanta m^{-2} s^{-1}) at 25 °C. Repeated addition of neutralized sulfide solution was used to obtain high cell yields (Siefert & Pfennig, 1984). Stock cultures were stored at 5 °C in the dark. Growth was followed photometrically by measuring optical density at 650 nm (UV/VIS spectrophotometer Lambda 2; Perkin Elmer).

Microscopic observations of cells of strain WS^T were done using a phase-contrast microscope (Axiophot; Zeiss). The fine structure of the cells was studied by electron microscopy after fixation of a cell pellet by the method of Ryter & Kellenberger (1958) and ultrathin sectioning of the cells. Observations were made with a JEOL 100 electron microscope.

The absorption spectrum of the living cells was measured after suspension of a cell pellet in 50 % glycerol using a UV/VIS spectrophotometer Lambda 2 (Perkin Elmer).

Growth tests were performed using Pfennig's medium described above aliquotted into 20 ml screw-capped tubes. According to the test conditions, the pH and the salt concentration were varied. Different salt concentrations were obtained using a concentrated salt solution containing (l^{-1}) 294 g NaCl and 47 g $MgCl_2.6H_2O$ (N. Pfennig, personal communication). The optimal pH was determined first and experiments to determine the optimal salt concentration were performed at optimal pH. Additional tests were performed at optimal pH and salt concentration. For nutritional experiments, several electron donors and carbon sources were tested, according to the recommended standards for the description of novel species (Imhoff & Caumette, 2004), with the final concentrations indicated in Table 1. The tubes were inoculated with a volume of 5 % preculture and incubated at 25 °C and 2000 lx (42 μmol quanta s^{-1} m^{-2}) for 5 days. For each experiment, three serial repetitions were carried out. Bacterial growth was measured as OD_{650} as described above. The measurements were performed using sterile Pfennig's medium as a blank and reference sample. Bacterial growth in standard Pfennig's medium incubated under exactly the same conditions was used as control.

To determine the possibilities of chemotrophic growth in the dark and growth in the presence of oxygen, tubes with 3 ml soft agar (1·8 %) were mixed with 6 ml medium, inoculated with 1 ml well-grown liquid culture and incubated with a headspace of air under both light (2000 lx, 42 μmol quanta s^{-1} m^{-2}) and dark conditions at 25 °C. The requirement for vitamin B_{12} as a growth factor was tested in medium free of vitamins and growth factors.

DNA from pure cultures was extracted using the QIAamp DNA Mini kit (Qiagen). The 16S rRNA gene was amplified using eubacterial primers 5′-27F (5′-AGTTTGATCCTGG-CTCAG-3′) and 3′-1492R (5′-GGTTACCTTGTTACGAC-TT-3′) and puReTaq Ready-To-Go PCR beads (Amersham Biosciences). The QIAquick PCR purification kit (Qiagen) was used to purify the PCR products. Sequence data were obtained using the method of Sanger *et al.* (1977). Automated sequence determination was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The complete sequence was assembled from several fragments by using the software SeqMan II 4.03 (DNASTAR) (Swindell & Plasterer, 1997).

For phylogenetic classification, alignments including various sequences from databases were created with the aid of the software program CLUSTAL X 1.83 (Thompson *et al.*, 1997). PHYLIP version 3.63 (Felsenstein, 2004) was used to create a distance matrix (based on the maximum-likelihood algorithm) and a phylogenetic tree was constructed with PHYML. For the determination of DNA base composition, the DNA was isolated by applying the method of Marmur (1961). The DNA base composition was determined according to Owen *et al.* (1969).

Under optimal growth conditions in the medium described above, single cells of strain WS^T exhibit a coccoid morphology. During binary fission, diplococci are formed. The cells are motile, have a mean cell diameter of 1·85 μm and do not contain gas vesicles (Fig. 1). Around individual cells, slight slime production is visible. In phases of stationary growth, cells form microcolonies of irregular shape (Fig. 1). When growing with sulfide and thiosulfate as photosynthetic electron donors, the cells contain sulfur globules stored inside the cell (Figs 1 and 2a). Electron microscopy of thin sections revealed the presence of an intracellular membrane system of the vesicular type and a cell wall typical of Gram-negative bacteria (Fig. 2). The external layer of the cell wall exhibits a multilayered structure (Fig. 2b).

Table 1. Morphological and physiological properties of strain WS^T and related reference strains

Strains: 1, *Thiorhodococcus mannitoliphagus* sp. nov. WS^T (data from this study); 2, *Trc. minor* ATCC 700259^T (Guyoneaud *et al.*, 1997); 3, '*Trc. drewsii*' DSM 15006 (Zaar *et al.*, 2003); 4, *Thiocystis violacea* DSM 207^T (unless indicated, data from Zaar *et al.*, 2003). ND, No data available; V, variable (strain dependent); +++, outstanding increase in growth to nearly 300 % of control; ++, increase in growth to >200 % of control; +, increase in growth to 150–200 % of control; (+), increase in growth to <150 % of control; −, no utilization (growth similar to control); (i), decrease in growth of up to 50 % compared with control; i, decrease in growth of >50 % compared with control. The following substrates (final concentration in mM in parentheses) were tested but were not used by strain WS^T: citrate (5), benzoate (2), sucrose (5), trehalose (5), glutamate (5), aspartate (5), gluconate (5), glycine betaine (5). Substrates tested as i are: caprylate (2), palmitate (2), cysteine (2), methionine (2). Substrates tested as (i) are: tartrate (5), butanol (5), glycerol (5), thioglycolate (2). All strains have spherical cells with flagellar motility and lack gas vesicles. All strains use sulfide, sulfur and thiosulfate as photosynthetic electron donors and photoassimilate acetate, propionate, pyruvate and fumarate as organic substrates.

Characteristic	1	2	3	4
Cell diameter (μm)	1·5–2·0	1·0–2·0	2·0–3·5	2·5–3·0
Colour of cell suspension	Purple–violet	Brown–orange	Brown–red	Purple–violet
Major carotenoid	Rhodopinal	Rhodopin	Rhodopin	Rhodopinal
DNA G+C content (mol%)	61·8	66·9	64·5	63·1
Salt optimum (% NaCl)	0·5–2·0	2·0	2·4–2·6	0–2·0
pH optimum	7·0–7·5	7·0–7·2	6·5–6·7	7·0–7·3
Temperature optimum (°C)	25–30	30–35	30–35	25–35
Light intensity optimum	2000 lx	2000 lx	50 μmol m^{-2} s^{-1}	ND
Vitamin requirement	B_{12}	No	No	No
Microaerobic growth	No	Yes	ND	Yes
Chemotrophic microaerobic growth in the dark	No	Yes	No	Yes
Utilization of substrates for growth*				
Sulfite (0·5 mM)	+	(2 mM) i	(2 mM) −	V
Formate (2 mM)	−	(5 mM) −	(5 mM) +	−†
Butyrate (5 mM)	−	−	+	+†
Valerate (5 mM)	(i)	−	+	ND
Lactate (5 mM)	+	++	−	−
Succinate (5 mM)	++	++	+	V
Malate (5 mM)	+	(+)	+	ND
Oxoglutarate (2 mM)	(+)	(5 mM) −	(5 mM) −	V
Glucose (5 mM)	++	−	−	V
Fructose (5 mM)	(+)	++	+	V
Glycolate (5 mM)	(i)	++	+	−†
Methanol (5 mM)	(i)	−	+	−†
Ethanol (5 mM)	i	++	+	−
Propanol (5 mM)	(i)	+	+	V
Mannitol (5 mM)	+++	−	−	ND
Peptone (0·05 %)	+	−	(0·025 %) −	ND
Casamino acids (0·05 %)	+	−	+	ND
Yeast extract (0·05 %)	+	+	(0·005 %) −	ND

*Tested at the concentration shown unless indicated.
†Data from Imhoff (2001).

Cell suspensions of liquid cultures of strain WS^T exhibit a purple–violet colour. The absorption spectrum showed maxima at 888, 856, 805, 591, 528, 493, 458 and 375 nm (Fig. 3), which indicates the presence of bacteriochlorophyll *a* and carotenoids of the rhodopinal series.

Growth of strain WS^T was decreased strongly below pH 7·0 and decreased slightly above pH 7·5 (Fig. 4a). The pH optimum is 7·0–7·5 and the pH tolerance is between 7·0 and 8·5. Growth experiments concerning the salt concentration revealed slightly decreased growth of strain WS^T at concentrations higher then 2 % NaCl and in strongly decreased growth with more than 3 % NaCl. No growth occurred at 5 % NaCl. Without salt, strain WS^T exhibited no growth, but growth increased very steeply at 0·1 % NaCl (Fig. 4b). Thus, the salt optimum of strain WS^T lies between 0·5 and 2·0 %, while the tolerance range can be defined as 0·1–3·0 %. Growth optima for temperature

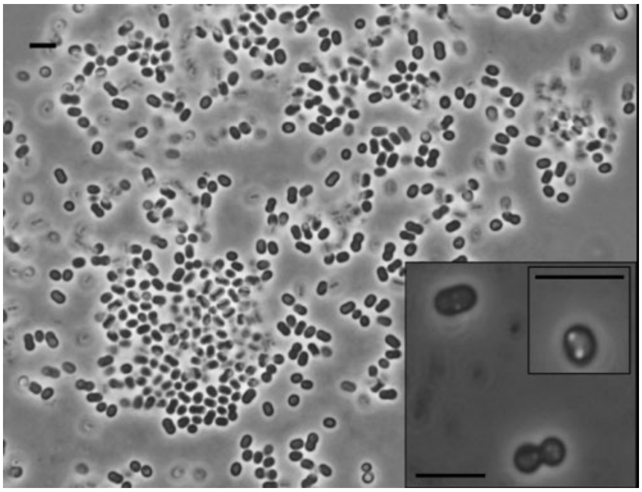

Fig. 1. Phase-contrast photomicrographs showing the morphology of strain WS^T. Cells were grown photolithoautotrophically with sulfide and thiosulfate under optimal conditions. Some cells show intracellular sulfur globules. Bars, 5 μm.

and light were found at 25–30 °C and 2000 lx (42 μmol quanta s^{-1} m^{-2}).

For photolithoautotrophic growth of strain WS^T under anoxic conditions in the light, sulfide, thiosulfate, sulfite and elemental sulfur were used as electron donors. Globules of elemental sulfur were formed during oxidation of sulfide and thiosulfate and stored transiently inside the cells. In the presence of carbonate and a reduced sulfur source (sulfide or thiosulfate or both), a number of organic substrates were photoassimilated. Strain WS^T exhibited photoassimilation of acetate, lactate, pyruvate, malate, peptone, Casamino acids and yeast extract, propionate, succinate, fumarate and glucose, while the utilization of mannitol resulted in an outstanding increase of growth. Oxoglutarate and fructose increased cell yields only slightly. No growth of strain WS^T was observed either under microaerobic conditions in the light or chemotrophically in the dark. Thus, the bacterium is strictly anaerobic and obligately phototrophic. Vitamin B_{12} is a required growth factor.

Analysis of the 16S rRNA gene sequence was used to reveal the phylogenetic placement of strain WS^T among other species of the family *Chromatiaceae*. The data clearly show that strain WS^T belongs to the genus *Thiorhodococcus* (Fig. 5). The highest sequence similarities found were to *Trc. minor* $CE2203^T$ (97·3 %) and '*Thiorhodococcus drewsii*' DSM 15006 (96·1 %). The base composition of purified DNA of strain WS^T is 61·8 mol% G+C.

Analysis of genetic relationships on the basis of 16S rRNA gene sequences, enabled by the establishment of sequencing techniques, demonstrated the ambiguous impact of morphological and physiological properties for the differentiation of bacterial species (Imhoff *et al.*, 1998b).

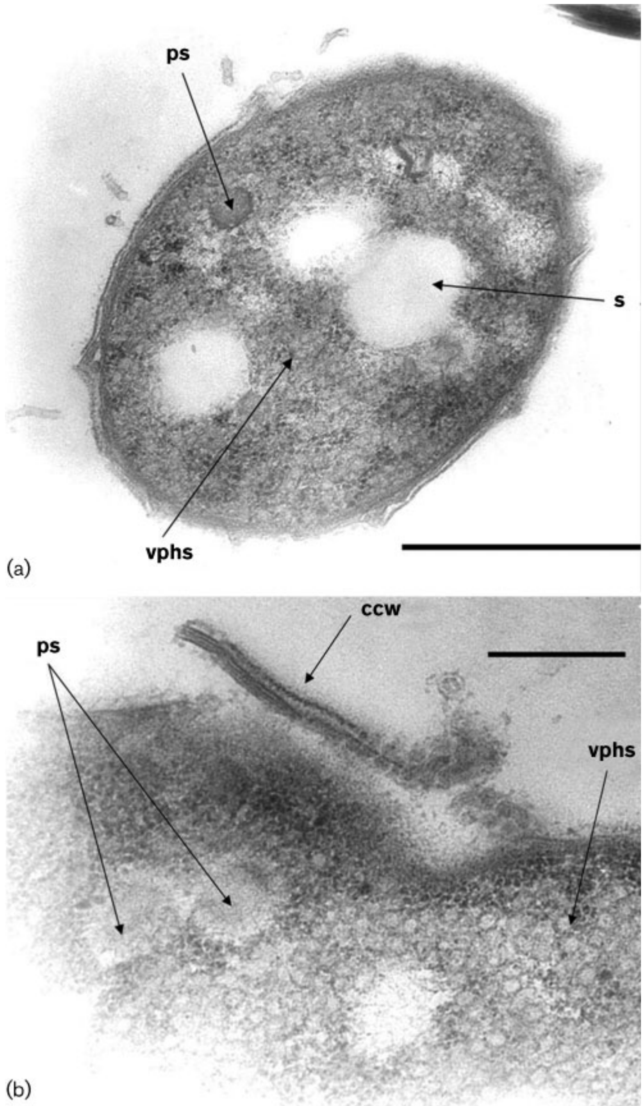

Fig. 2. Electron photomicrographs of ultrathin sections of cells of strain WS^T grown photoautotrophically. Vesicular internal membranes have developed within the bacteria. (a) Whole-cell ultrathin section showing sulfur globules. (b) Detailed ultrathin section of a cell fragment showing photosynthetic membrane system of the vesicular type and the multilayered structure of the cell wall. Abbreviations: ps, polysaccharides; vphs, vesicular type of photosynthetic structures; s, sulfur inclusions; ccw, outer layer of cell wall. Bars, 1 μm.

Consequently, the taxonomy of phototrophic bacteria has been carefully revised by combining information from gene sequences and selected phenotypic characteristics as diagnostic properties (Imhoff & Süling, 1996; Imhoff *et al.*, 1998a, b; Imhoff, 2003; Guyoneaud *et al.*, 1998). By combining genetic and phenotypic features, clear differentiation of the novel bacterial species described in this communication from closely related species was achieved. Morphological properties such as cell shape and size, the absence of

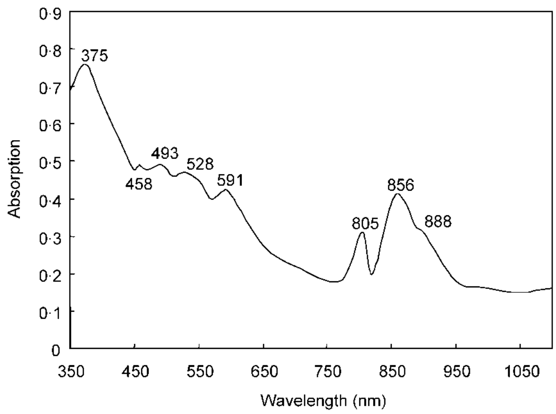

Fig. 3. Absorption spectrum of living cells of strain WS^T suspended in 50 % glycerol.

gas vesicles and motility of the new isolate are in accordance with properties of the genus *Thiorhodococcus* as well as *Thiocystis*. Genetically, strain WS^T is affiliated to the genus *Thiorhodococcus*. Experimental studies revealed significant phenotypic differences from related *Thiorhodococcus* species (Table 1) as well as clear separation by 16S rRNA gene sequence similarities from both *Trc. minor* (97·3 % similarity to the type strain) and '*Trc. drewsii*' (96·1 % similarity to the proposed type strain). Both *Trc. minor* and '*Trc. drewsii*' contain spirilloxanthin as main carotenoid, while rhodopinal is the major component in strain WS^T. With respect to physiological properties, slight differences can be seen in pH and salt requirements. Compared with other species of this genus, the salt optimum of strain WS^T is shifted slightly towards lower salt concentrations. This property is probably connected with the isolation of the novel strain from microbial mats developing in brackish, shallow supralittoral zones of the White Sea. On the other hand, the pH requirement of strain WS^T exhibits an affinity to slightly more alkaline conditions (pH 7·5 and 8·5). In terms of substrate utilization, again similarities and differences can be seen. Common to all members of the genus *Thiorhodococcus* is the utilization of acetate, pyruvate, succinate, malate and fumarate as carbon substrates. Glucose is used exclusively by strain WS^T. An outstanding and unique characteristic of strain WS^T is good growth with mannitol.

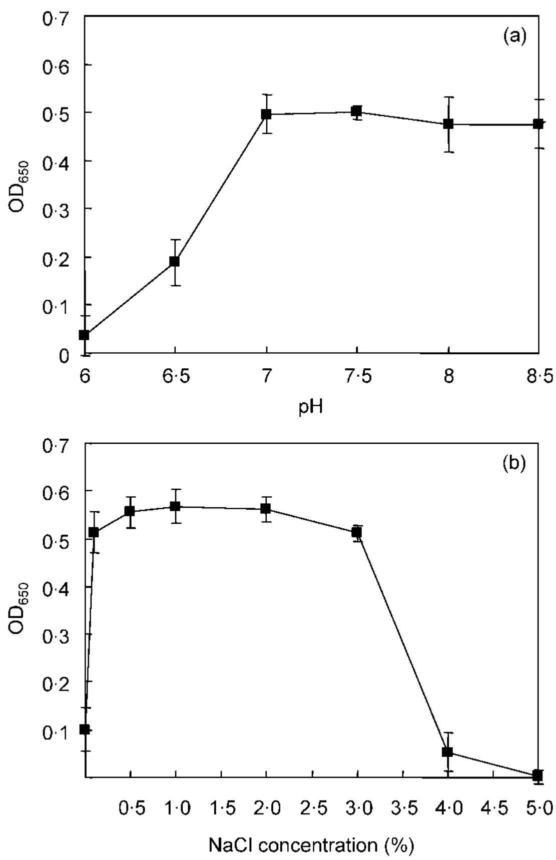

Fig. 4. Influence of pH (a) and salt concentration (b) on growth of strain WS^T. Experiments to determine the effect of salt concentration were performed at optimal pH.

Description of *Thiorhodococcus mannitoliphagus* sp. nov.

Thiorhodococcus mannitoliphagus (mann.i.to′li.pha′gus. N.L. n. *mannitolum* mannitol; Gr. v. *phagein* to eat; N.L. masc. adj. *mannitoliphagus* consuming mannitol).

Cells are coccoid with mean cell diameter of 1·85 μm. During binary fission, diplococci are formed. Cells are Gram-negative, motile by flagella and do not contain gas vesicles. In the stationary growth phase, cells form irregular microcolonies. Colour of cell suspensions is purple–violet. Photosynthetic membrane system is of the vesicular type. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the rhodopinal series. Phototrophic growth occurs under anoxic conditions in the light. No growth occurs under microaerobic conditions in the light or under chemotrophic conditions in the dark. Vitamin B_{12} is required as a growth factor. Electron donors used for photolithoautotrophic growth are sulfide, thiosulfate, sulfite and elemental sulfur. Globules of elemental sulfur, which are formed during photolithoautotrophic growth with sulfide and thiosulfate, are stored transiently inside the cells and are oxidized further to sulfate. In the presence of carbonate and a reduced sulfur source (sulfide and/or thiosulfate), oxoglutarate, fructose, acetate, lactate, pyruvate, malate, peptone, Casamino acids, yeast extract, propionate, succinate, fumarate, glucose and mannitol are photoassimilated. The utilization of mannitol results in an outstanding increase in growth. Conditions for optimal growth are 25–30 °C, 2000 lx (42 μmol m^{-2} s^{-1}), pH 7·0–7·5 and concentrations of 0·5–2 % NaCl. The DNA base composition of the type strain is 61·8 mol% G + C.

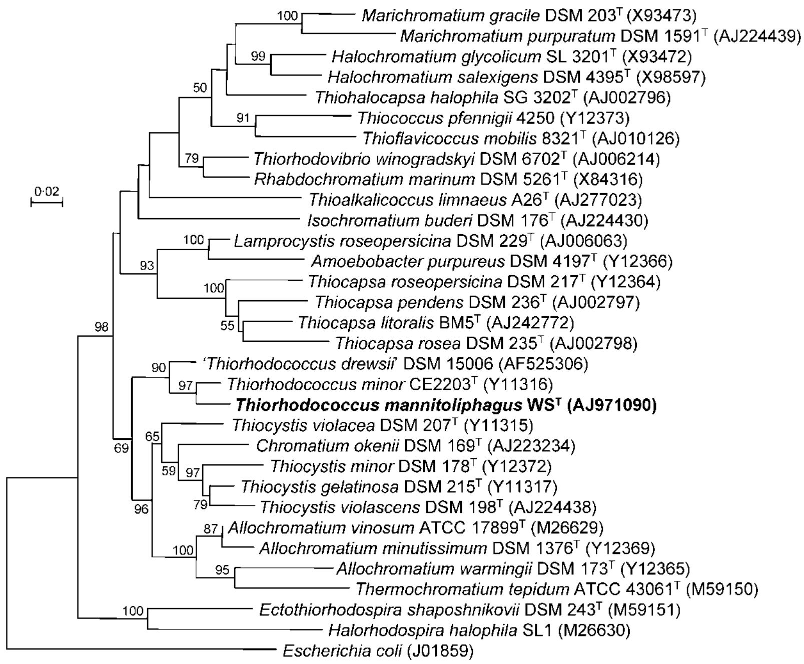

Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences illustrating the relationship of *Thiorhodococcus mannitoliphagus* WS^T and related purple sulfur bacteria. Calculations were done as described in the text. Bar, 0·02 substitutions per position.

The type strain, WS^T (=ATCC BAA-1228^T=VKM B-2393^T), was isolated from microbial mat communities of an estuary of the White Sea.

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